Metabolic regulation of leaf respiration and alternative pathway activity in response to phosphate supply

M. A. GONZÁLEZ-MELER, L. GILES, R. B. THOMAS & J. N. SIEDOW

Introduction

Plant respiration serves two important metabolic functions. It provides carbon intermediates used in the synthesis of carbohydrate and ATP. The biochemical basis of the alternative pathway is an oxidase in the mitochondrial electron transport chain that transfers electrons from reduced ubiquinone to O₂, bypassing two sites of proton translocation and releasing the resulting free energy as heat (Moore & Siedow 1991). The activity of the alternative pathway is thought to be dependent on: (i) the level of its substrate, reduced ubiquinone; (ii) the level of alternative oxidase protein present; and (iii) the activation state of the alternative oxidase protein.

Abstract

In this study the question whether the alternative respiratory pathway acts as an electron bypass for the cytochrome pathway under conditions of growth on limited phosphorus was investigated. The oxygen isotope fractionation technique was used to assess the in vivo activities of the cytochrome and alternative respiratory pathways in the absence of added inhibitors. The response of respiration to low phosphorus supply varied among species. Growth at low phosphorus reduced cytochrome pathway activity in bean and tobacco. Alternative pathway activity increased only in bean leaves in response to low phosphorus and not in tobacco. In the case of *Gliricidia sepium*, cytochrome pathway activity remained unchanged whereas the alternative pathway activity increased with low nutritional phosphorus. At low phosphorus, alternative oxidase protein levels increased in the leaves of bean and *G. sepium* but not in tobacco, suggesting a dependence of alternative pathway activity on protein level. Alternative pathway activity was also not correlated with soluble carbohydrate concentration in bean or tobacco at any phosphorus level. These results show that the alternative pathway does not always act as an electron bypass in response to the downstream restriction of the cytochrome pathway imposed by low phosphorus supply. These results suggest that factors in addition to cellular carbohydrate level and adenylate control can act to regulate alternative pathway activity.

Key-words: *Gliricidia sepium*, *Nicotiana tabacum*, *Phaseolus vulgaris*, alternative oxidase, cytochrome pathway, oxygen isotope fractionation, regulation of respiration, respiration.
Parsons 1999). Increases in protein level alone do not always result in increased alternative pathway activity (Lennon et al. 1997; González-Meler et al. 1999). This may be explained by the fact that alternative oxidase activity is subject to post-translational regulation involving the reduction state of a regulatory sulphhydryl-disulphide system (Umbach & Siedow 1993) and activation by α-keto acids, such as pyruvate (Millar et al. 1993; Umbach et al. 1994; Rhoads et al. 1998). Characterizing these regulatory features also led to recognition that the alternative pathway can readily compete for electrons with an unsaturated cytochrome pathway (Ribas-Carbo et al. 1995), so the alternative pathway does not always require the presence of ‘excess’ carbohydrate to operate. Therefore, to identify its possible physiological role it is important to better understand how alternative pathway activity is controlled in vivo.

Phosphorus limitation can provide information on the regulation of respiration and alternative pathway activity. Phosphorus deficiency limits whole-plant photosynthesis (Heineke, Stitt & Heldt 1989) and, as a consequence, growth is reduced (Sivak & Walker 1986). Despite the dramatic effects of low-phosphorus supply on plant size, specific rates of photosynthesis may (Jacob & Lawlor 1993; Rao, Fredeen & Terry 1993) or may not (Foyer & Spencer 1986; Kondracka & Rychter 1997) be affected. Therefore, specific leaf respiration at low phosphorus may not be limited by carbohydrate availability. Phosphorus limitation also induces metabolic adaptations in plants that can bypass the adenylate-phosphate-dependent reactions of glycolysis and respiration (Duff et al. 1989; Rychter & Mikulska 1990; Theodorou et al. 1991; Hoefnagel, van Iren & Libbenga 1993; Theodorou & Plaxton 1993; Plaxton & Carswell 1999), including the cytochrome pathway. The alternative pathway can act as such a bypass of phosphate control on electron transport (Theodorou & Plaxton 1993). Moreover, restriction of the cytochrome pathway by phosphate deficiency could result in over-reduction of the ubiquinone (UQ)-pool if the alternative oxidase is not active, generating harmful reactive oxygen species (Wagner & Moore 1997; Parsons, Yip & Vanlerbergh 1999). So, although phosphate limitation can inhibit electron transport through the cytochrome pathway, it may simultaneously enhance the activity of the alternative pathway (Rychter & Mikulska 1990; Rychter et al. 1992; Mikulska, Bomsel & Rychter 1998). Following phosphorus limitation, respiration in the presence of cyanide, referred to as ‘alternative pathway capacity’, has been reported to increase (Rychter & Mikulska 1990; Rychter et al. 1992; Hoefnagel et al. 1993; Mikulska et al. 1998). Some of these studies also suggested that alternative pathway activity increased under low phosphorus. However, such conclusions were based on the use of respiratory inhibitors to assess its activity, which has been shown to be unreliable (Ribas-Carbo et al. 1995; Day et al. 1996).

To better understand the features that regulate respiration and alternative pathway activity in vivo, we have investigated the response of respiration in the leaves of three species (Phaseolus vulgaris, Nicotiana tabacum, Gliricidia sepium) to low phosphorus supply.

**MATERIALS AND METHODS**

**Plant material**

*Phaseolus vulgaris* (L.), cv Blue Lake bush 274, seeds were germinated in 10 L pots filled with acid-washed sand. After the plants had consumed their endogenous phosphate reserves (15 d, Sharkey & Vandermeer 1989), they were supplemented daily with a nutrient solution containing either 0·005 mm (low) or 1·0 mm (control) P added as K₂HPO₄ and KH₂PO₄. Nutrient solutions were adjusted to pH 6·5 and were completed with 4·5 mm N (NO₃), 5·0 mm K, 3·5 mm Ca, 1·5 mm Mg, 2·0 mm S, 1·0 mm Na, 0·16 mm Fe, 0·05 mm B, 0·01 mm Mn, 0·001 mm Zn, 0·001 mm Cu, 0·05 μm Mo, 0·16 μm Co and 0·04 mm Fe-EDTA. The plants were grown in the Duke Phytotron greenhouses between May and July 1999, with an average light of 25 mol m⁻² d⁻¹ and day and night mean temperatures of 28 and 21 °C, respectively. Fully developed leaf samples were harvested between 8 and 11 weeks after germination depending on Pi conditions.

*Nicotiana tabacum* L., cv Petit Havanna SR1 plants were grown in 9 L pots filled with acid-washed sand in growth chambers. After the plants had consumed their endogenous phosphate reserves for 1 week, they were supplemented twice weekly with a nutrient solution containing either 0·10 mm (low) or 1·25 mm (control) P added as K₂HPO₄ and KH₂PO₄ (Paul & Stitt 1993). Nutrient solutions were adjusted to pH 6·5 and contained 10 mm N (NO₃), and additional nutrients as described above for *P. vulgaris*. Growing conditions were 16 h light at 600 μmol m⁻² s⁻¹ at 25 °C and 8 h of dark at 20 °C. Relative humidity was maintained at 70%. Fully developed leaf samples were harvested between 7 and 11 weeks after germination depending on Pi conditions.

Seeds of the tropical shrub *Gliricidia sepium* (Jacq.) Walp. from a single provenance, 19°40′N, 155°55′ W, elevation 200 m (Agroforester Tropical Seeds, Holualoa, HI, USA) were germinated in 3·3 L pots filled with acid-washed sand. All seeds were inoculated before planting by wetting a 1 : 9 (v/v) solution containing three strains of *Rhizobium* spp. (Agroforester Tropical Seeds). Plants were watered to saturation each morning and afternoon with nutrient solutions containing either 0·005 mm (low) or 0·50 mm (control) P added as KH₂PO₄. Nutrient solutions were adjusted to pH 6·1 and were otherwise completed with 1·0 mm N (NO₃), 3·4 mm K, 3·5 mm Ca, 1·5 mm Mg, 3·0 mm S, 0·16 mm Fe, 0·05 mm B, 0·01 mm Mn, 0·001 mm Zn, 0·001 mm Cu, 0·05 μm Mo and 0·16 μm Co. A combination of high pressure sodium vapour and metal halide high-intensity discharge lamps provided a photosynthetic photon flux density of 1100 μmol m⁻² s⁻¹. Plants were grown under a 14 h light : 10 h dark photo- and 28/23 °C thermoperiod. Relative humidity was approximately 70% during the day. Fully developed leaf samples were harvested between 12 and 16 weeks after germination depending on Pi conditions.

At the time of sampling, phosphorus deficiency reduced plant growth and individual and total plant leaf area and mass for all species studied consistent with other studies...
made on the same species (Paul & Stitt 1993; Kondracka & Rychter 1997).

Respiratory measurements and oxygen isotope analysis

We analysed total, cytochrome and alternative respiratory activities of leaves using oxygen isotope fractionation. Samples of fully developed leaves (0.9–1.2 g fresh weight) were kept in the dark for 25 min at 23 °C before gas-phase respiratory measurements were taken in a closed 4.96 mL stainless-steel temperature-controlled cuvette at 23 °C (González-Meler et al. 1997). A CO₂ absorber (ascarite II) was present during measurements to avoid inhibition of respiration as a consequence of the build up of CO₂ in the closed cuvette during the course of the experiment (González-Meler et al. 1996). The CO₂ absorber was replaced after each measurement. Surface-dried leaf samples were placed in the cuvette and left with the inlet vent open for 3–5 min with air mixing to allow for air and isotopic equilibration between the inside and the outside of the tissue. Oxygen extraction and isotope analysis were carried out as described in Robinson et al. (1997) and González-Meler et al. (1999). At regular time intervals an air sample was taken into a 100 μL loop and directed into the helium flow of the gas chromatography–mass spectrometry unit. Water vapour and remaining carbon dioxide were removed and the oxygen, argon and nitrogen gases separated by gas chromatography (8619 A gas chromatograph; SRI Instruments Inc, Las Vegas, NV, USA) using a 914.4 mm × 6.35 mm molecular sieve MS 5 A (80–100 mesh) column (Alltech, Deerfield, IL, USA) heated to 50 °C at a flow rate of 30 mL min⁻¹ He carrier gas. The components were detected using a thermal conductivity detector and integrated using a Hewlett Packard (Model 3394) integrator. Because the column cannot resolve argon and oxygen separately, an argon-to-nitrogen gain factor of 1.066 was measured with the SRI gas chromatograph and used to correct the observed O₂ peak. The ²⁰O/¹⁸O isotope ratio of the sample was then measured directly from the ratio of masses 32 and 34 using a SIRA Series II isotope ratio mass spectrometer (VG ISOGAS, Middlewich, Cheshire, UK) operated in continuous flow mode. Calculations of oxygen isotope fractionation were made as described in Guy et al. (1989) with modifications (González-Meler et al. 1999).

The sampling system was checked regularly for diffuse leaks from external air by filling the cuvette with He and sampling the cuvette for appearance of air over time. Leaks were always very small and non-diffusive (i.e. oxygen isotopic composition of leaked air was equal to that of external air). Over the course of the experiment, each sample consumed at least 30% but no more than 50% of the initial oxygen. The r² values of all unconstrained linear regressions between ln(1/F) and ln(Ro/Ro) (with at least five data points) were greater than the value of 0.995 considered minimally acceptable (Ribas-Carbo et al. 1995, 1997, 2000a; Lennon et al. 1997; González-Meler et al. 1999). During inhibitor treatments, either 1 mM KCN (in 1 mM TES, pH 8.0) or 6 mM (10 mM for G. sepium leaves) SHAM (in water from a 1.0-m stock in dimethylsulphoxide) were applied by sandwiching the leaf tissues between medical wipes soaked with the corresponding inhibitor and incubating in the dark for at least 30 min. The inhibitor concentrations were obtained from titrations carried out in a liquid phase oxygen electrode and were similar to values used previously for these species (Lennon et al. 1997; González-Meler, Matamala & Peñuelas 1998b). All stocks were freshly prepared before use. Tissues were surface dried before being placed in the measurement cuvette. The CO₂ absorber was removed in experiments requiring KCN to avoid recovery from inhibition. In addition, for KCN experiments, a piece of tissue wetted with KCN was present in the cuvette. Electron partitioning between the two pathways in the absence of inhibitors was calculated as described by Guy et al. (1989).

Immunoblotting

Mitochondrial mini-preparations were performed as described by Lennon et al. (1997), and full mitochondrial isolations as described in Umbach & Siedow (1993). Protein concentrations were estimated by the method of Lowry et al. (1951).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed with mitochondria or tissue extracts using 10–17% gradient polyacrylamide gels. For alternative oxidase protein, samples were prepared with 100 mM DTT as a reductant in the sample buffer (Umbach & Siedow 1993; Umbach et al. 1994). Proteins were transferred to nitrocellulose according to Towbin, Staehelin & Gordon (1979), and immunoblotting was performed as described by Lennon et al. (1997) using the alternative oxidase monoclonal antibody (AOA) against the alternative oxidase protein from Sauromatum guttatum Schott (Elthon, Nickels & McIntosh 1989) at 1 : 100 dilution (1 : 100 for G. sepium). For the ATP synthase immunoblots, polyclonal antibodies against the β-subunit of the mitochondrial ATP synthase (F₁-ATP synthase) of Zea mays L. leaves were used at 1 : 1000 dilution. For the leaf phosphoenolpyruvate carboxylase (PEPCase) immunoblots, 0.5 μL of column affinity purified antibodies against PEPCase from suspension cells of Brassica napus L. were used at 1 : 30 dilution. Bound antibodies were detected using an antimouse (AOA) or antirabbit (F₁-ATP synthase, PEPCase) horseradish peroxidase conjugate at 1 : 25 000 dilution (1 : 50 000 for blots using tissue extracts) and alternative oxidase, F₁-ATP synthase and PEPCase bands were detected with a super-signal chemi-luminescence assay (DuPont NEN: DuPont, Boston, MA, USA) according to the manufacturer’s instructions. Densitometry to quantify the relative protein levels of the three enzymes was performed as described by Umbach & Siedow (1993).

Leaf chemical analysis

After the dry weight of the sample used for respiratory measurements was obtained (in a forced air oven at 65 °C
until constant dry weight was reached), samples were finely ground and a subsample of 15 mg was mixed with 2 mL of 12 : 5 : 3 methanol : chloroform : water (v/v) solution for soluble sugar and starch analysis (Tissue & Wright 1995). Samples were shaken for 30 min and then centrifuged for 4 min at 2500 g. The supernatant was saved and the procedure was repeated twice with the pellet. Supernatants were mixed and kept at 4 °C for separation of the soluble sugars in the water–methanol fraction from pigments and lipids (chloroform fraction). Dried pellets (hood dried) were mixed with 5 mL of 35% HClO4 to digest polysaccharides into soluble sugars for 2 h on a shaker at room temperature. Digested samples were filtered with Whatman no. 1 filter paper. For sugar and starch analysis, a 200 μL sub-sample was mixed with 1·8 mL of 3% phenol (in water) and 5 mL of concentrated H2SO4. Samples were read after 10 min in a spectrophotometer (Lambda, Perkin Elmer) at 490 nm against glucose and starch standards.

Subsamples (25 mg) of dried plant and reference material were digested at 320 °C in a sulphuric acid and hydrogen peroxide mixture (Kjeldahl digestion) for organic N and P analysis using a Technicon auto-analyser according to Lowther (1980) and Wolf (1982).

**RESULTS**

Effects of phosphorus nutrition on leaf respiration and oxygen isotope fractionation

The effect of phosphorus deficiency on the total respiratory rate and the activity of the cytochrome and alternative pathways in fully expanded leaves were assessed (Fig. 1). When compared with plants grown at full nutrient solution, respiration in leaves of *P. vulgaris* was reduced by 43% in the low phosphorus treatment (Fig. 1a; *P* < 0·01), and the respiratory rate of tobacco leaves was decreased by 32% (Fig. 1b; *P* < 0·001). In contrast, leaf respiration in low-phosphate-grown *G. sepium* increased 23% in comparison with high-phosphate-grown plants (Fig. 1c; *P* < 0·01).

Oxygen isotope fractionation by SHAM-resistant respiration (i.e. cytochrome pathway) was 19·5‰ ± 0·5, 19·6‰ ± 0·2 and 19·9‰ ± 0·2 for leaves of *P. vulgaris*, *N. tabacum* and *G. sepium*, respectively. The oxygen isotope fractionation by cyanide-resistant respiration (i.e. alternative pathway) was 30·3‰ ± 0·4, 29·8‰ ± 0·3 and 30·7‰ ± 0·8 for leaves of *P. vulgaris*, *N. tabacum* and *G. sepium*, respectively. The low phosphorus nutrition treatment did not influence the oxygen isotope fractionation by either the cytochrome or alternative pathways. These values are

<table>
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<th>High phosphorus</th>
<th>Low phosphorus</th>
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<tr>
<td><em>P. vulgaris</em></td>
<td>Fractionation (%)</td>
<td>20·3 ± 0·2</td>
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<td></td>
<td><em>τ</em></td>
<td>0·08 ± 0·02</td>
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<tr>
<td><em>N. tabacum</em></td>
<td>Fractionation (%)</td>
<td>20·5 ± 0·2</td>
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<tr>
<td></td>
<td><em>τ</em></td>
<td>0·09 ± 0·02</td>
</tr>
<tr>
<td><em>G. sepium</em></td>
<td>Fractionation (%)</td>
<td>21·3 ± 0·4</td>
</tr>
<tr>
<td></td>
<td><em>τ</em></td>
<td>0·13 ± 0·04</td>
</tr>
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Values are means ± standard error of three to five plant replicates.

* Statistical difference at *P* < 0·05 between plants grown at the two phosphorus levels, rank summary test.

similar to those previously reported for cytochrome and alternative pathway fractionation in green leaves (Lennon et al. 1997; González-Meler et al. 1999; Ribas-Carbo et al. 2000a, b) and mitochondria isolated from green tissues (Ribas-Carbo et al. 1995, 1997). Residual respiration (measured in the presence of both KCN and SHAM) in the three species studied was less than 7% of the uninhibited rate of respiration. Oxygen isotopic fractionation during leaf respiration in high-phosphorus-grown plants was slightly lower in P. vulgaris and N. tabacum than in G. sepium (Table 1). Growth in low-phosphorus increased the oxygen isotope fractionation compared to control plants (P < 0.01). Electron partitioning to the alternative pathway was 13 and 28% of total respiration for plants grown at high and low phosphorus, respectively (Table 1; P < 0.05). In G. sepium, over 90% of the increase in total leaf respiration in plants grown at low phosphorus was due to the increased activity of the alternative pathway.

Respiratory protein levels

Immunoblotting was used to assess the relative abundance of the alternative oxidase in mitochondria isolated from leaves of the plants used for the respiratory studies detailed above. Low phosphorus supply increased the level of alternative oxidase protein 2.5-fold in mitochondria isolated from leaves of P. vulgaris compared to plants grown with full nutrients (Fig. 2a). However, in tobacco leaves, the level of alternative oxidase protein was not changed by the level of nutritional phosphate (Fig. 2a). In leaves of G. sepium, the amount of alternative oxidase protein was 40% higher in mitochondria from plants grown at low phosphorus compared to high-phosphorus-grown plants (Fig. 2a). The β-subunit of the mitochondrial F1-ATP synthase was not affected by phosphorus levels in either P. vulgaris or N. tabacum (Fig. 2b). However, the levels of F1-ATP synthase

Figure 2. Immunoblots of the alternative oxidase (Alt Ox; a), the β-subunit of the F1-ATP synthase (ATPase; b), the PEPCase (c), and leaf cyanide-resistant respiration rates (VKCN) from leaves of P. vulgaris, N. tabacum and G. sepium grown at high and low phosphorus. For the alternative oxidase, DTT-treated mitochondrial protein equivalent to 40 μg was loaded on each lane. For the β-subunit of the F1-ATP synthase, mitochondrial protein equivalent to 20 μg was loaded on each lane. For the PEPCase, tissue extract equivalent to 20 μg fresh mass was loaded on each lane. Cyanide-resistant respiration is an average of three to four replicates and measured as μmol O2 kg dry mass s−1 from the same tissue used for isotope fractionation experiments. Standard errors were less than 10% of the mean value.
in *G. sepium* increased slightly in plants grown at low phosphorus compared with control plants.

Increases in alternative oxidase protein levels in mitochondria are often reflected in increases in the rate of tissue cyanide-resistant respiration (see González-Meler et al. 1999 for recent discussion). In leaves of *P. vulgaris* cyanide-resistant respiration increased from 19 μmol O₂ kg⁻¹ DM s⁻¹ in control plants to 38 μmol O₂ kg⁻¹ DM s⁻¹ in plants grown at low phosphorus, consistent with the 2.5-fold increase in protein level (Fig. 2a; *P* < 0.05). There was no change in the cyanide-resistant respiration rate in tobacco leaves (11 and 12 μmol O₂ kg⁻¹ DM s⁻¹ for high- and low-phosphate-grown plants, respectively). Leaves of *G. sepium* grown at limited phosphorus did not increase their cyanide-resistant respiration rate (3.0 μmol O₂ kg⁻¹ DM s⁻¹) relative to control plants (3.3 μmol O₂ kg⁻¹ DM s⁻¹), despite the fact that alternative oxidase protein level increased somewhat in plants grown at low phosphorus.

Phosphoenolpyruvate carboxylase (PEPCase) has been shown to increase in plants grown at low phosphorus (Duff et al. 1989; Plaxton 1996). The levels of PEPCase in leaves of *P. vulgaris* grown at low phosphorus increased markedly (> four-fold) when compared to plants grown at full nutrition (Fig. 2c). Similarly PEPCase in leaves of tobacco plants grown at low phosphorus increased over five-fold when compared with plants grown with sufficient phosphorus.

### Leaf chemical composition

In comparison with control plants, *P. vulgaris* leaf starch and soluble sugar concentrations decreased 38% (*P* < 0.05) and 30% (*P* < 0.01), respectively, in plants grown at low phosphorus (Table 2; *P* < 0.05). In *N. tabacum*, starch levels decreased 41% (*P* < 0.05) but sugar levels increased 50% (*P* < 0.05) in plants grown at low phosphorus (Table 2). Starch levels decreased 25% (*P* < 0.05) in *G. sepium* leaves grown under limited phosphorus nutrition, but soluble sugar levels were not affected (Table 2).

The levels of leaf organic N were higher in *P. vulgaris* grown at low phosphorus when compared with plants grown at high phosphorus (Table 2; *P* < 0.001). There were no differences in organic N composition in leaves of *N. tabacum* and *G. sepium* between high- and low-phosphorus-grown plants. The levels of leaf organic phosphorus were substantially lower in low-phosphorus-grown plants for all three species (Table 2; *P* < 0.01).

### Mitochondrial ATP production

The rate of mitochondrial ATP synthesis can be calculated from the activities of the cytochrome and alternative pathways, assuming that electron flow through the alternative pathway promotes some synthesis of ATP via complex I and some proton leakage, as described by Amthor (1994). Mitochondrial ATP synthesis in low-phosphorus-grown plants was 50 and 28% lower than controls in leaves of *P. vulgaris* (*P* < 0.01) and *N. tabacum* (*P* < 0.05), respectively (Table 3). The rate of ATP synthesis in leaves of *G. sepium* was marginally higher in low-phosphorus-grown plants (10%; *P* < 0.05), due to the increased respiratory activity via the alternative pathway (Table 3).

In fully expanded leaves, respiration is often correlated

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### Table 2. Starch, soluble carbohydrate, organic N and organic P content in leaves of plants grown at either high- or low-phosphorus

<table>
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<th>Species</th>
<th>High Pi</th>
<th>Low Pi</th>
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<tr>
<td><strong>Starch</strong> (mg g⁻¹ DM)</td>
<td>220 ± 32</td>
<td>137 ± 5*</td>
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<tr>
<td><strong>Sugar</strong> (mg g⁻¹ DM)</td>
<td>59 ± 7</td>
<td>41 ± 4*</td>
</tr>
<tr>
<td><strong>Organic N</strong> (mg g⁻¹ DM)</td>
<td>40 ± 2</td>
<td>64 ± 4*</td>
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<tr>
<td><strong>Organic P</strong> (mg g⁻¹ DM)</td>
<td>2.9 ± 0.5</td>
<td>1.2 ± 0.2*</td>
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Values are means ± standard error of three to six replicates.

* Statistical difference at *P* < 0.05 between plants grown at the two phosphorus levels, rank summary test.

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### Table 3. Maximum rates of mitochondrial ATP synthesis calculated from respiration and oxygen isotope fractionation measurements on a dry mass or tissue organic N basis in leaves of plants grown at either high- or low-phosphorus

<table>
<thead>
<tr>
<th>Species</th>
<th>High Pi</th>
<th>Low Pi</th>
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<tbody>
<tr>
<td><strong>ATP (μmol kg⁻¹ DM s⁻¹)</strong></td>
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<tr>
<td><em>P. vulgaris</em></td>
<td>205 ± 13</td>
<td>101 ± 18*</td>
</tr>
<tr>
<td><em>N. tabacum</em></td>
<td>116 ± 7</td>
<td>83 ± 11*</td>
</tr>
<tr>
<td><em>G. sepium</em></td>
<td>53 ± 2</td>
<td>59 ± 1*</td>
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<th>Species</th>
<th>High Pi</th>
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<tbody>
<tr>
<td><strong>ATP (μmol g⁻¹ N s⁻¹)</strong></td>
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<tr>
<td><em>P. vulgaris</em></td>
<td>5.2 ± 0.5</td>
<td>1.7 ± 0.3</td>
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<tr>
<td><em>N. tabacum</em></td>
<td>5.2 ± 0.2</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td><em>G. sepium</em></td>
<td>ND</td>
<td>ND</td>
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</table>

Values are means ± standard error of three to six replicates.

* Statistical difference at *P* < 0.05 between plants grown at the two phosphorus levels, rank summary test.

ND, not determined.
with tissue N content (Amthor 1989; Cannell & Thornley, 2000). Respiratory ATP production, based on unit of tissue nitrogen in bean plants grown at low phosphorus, was three-fold lower than in plants grown at high phosphorus (Table 3), due in part, to the increase in tissue N content (Table 2). However, the concentration of N in tobacco leaves was lower in plants grown at low phosphorus in comparison with plants grown at high phosphorus, so ATP production per unit of tissue N was the same in plants grown at low or high phosphorus (Table 3).

**DISCUSSION**

The response of the cytochrome and alternative pathways to growth at low phosphorus varied among species (Fig. 1). The alternative pathway has been suggested to act as a bypass for the cytochrome pathway under conditions of limited oxidative phosphorylation (Plaxton & Carswell 1999). Despite the fact that plant growth at low phosphorus reduced cytochrome pathway activity in both bean and tobacco leaves, the alternative pathway activity only increased with bean. Moreover, in *G. sepium*, alternative pathway activity increased at low nutritional phosphorus with no apparent inhibition of the cytochrome pathway. These results show that any bypass role of the alternative pathway in response to restricted cytochrome pathway activity is species-dependent, at least during a long-term response to phosphorus starvation.

Increases in alternative oxidase protein levels are often correlated with increases in cyanide-resistant respiration rates (Obenland et al. 1990; Fiorani, Millenaar & Lambers 1998; González-Meler et al. 1999). Bean leaves grown at low, growth-limiting phosphorus showed an increase in alternative oxidase protein levels (Fig. 2a), which was accompanied by an increased rate of cyanide-resistant respiration, consistent with previous observations (Rychter & Mikulska 1990). Tobacco leaves grown at limiting phosphorus showed no altered alternative oxidase protein level or cyanide-resistant respiratory rate compared with high-phosphorus-grown plants (Fig. 2a). This differs from results obtained using heterotrophic tobacco cell culture where the levels of alternative oxidase protein increased when phosphorus limited cell growth (Parsons et al. 1999). This indicates that differences exist in the metabolic responses of intact plants and cell cultures and that the latter may not be a good model system for establishing alternative oxidase responses in intact plants. Leaves of *G. sepium* grown at low phosphorus showed some increased alternative oxidase protein but no change in tissue cyanide-resistant respiratory activity. With regard to alternative pathway activity in the absence of added inhibitors, in both instances of increased activity at low phosphorus (*P. vulgaris* and *G. sepium*) there was also a concomitant increase in alternative oxidase protein level.

The alternative pathway has been proposed to operate as a bypass of the ADP- and Pi-controlled cytochrome pathway during phosphorus limitation (Theodorou & Plaxton 1993; Parsons et al. 1999). Phosphorus deprivation is known to induce phosphorus- and adenylate-independent enzymes in the cytosol, bypassing major control points of glycolysis and allowing carbon metabolism to continue (Duff et al. 1989; Plaxton 1996; Plaxton & Carswell 1999). Among these bypasses is the induction of PEPCase, which can circumvent pyruvate kinase (Plaxton 1996; Plaxton & Carswell 1999). The levels of PEPCase increased in the leaves of both bean and tobacco grown at low-phosphorus (Fig. 2c). However, upregulation of PEPCase at low phosphorus was not accompanied by an increase in alternative oxidase protein in tobacco leaves (Fig. 2), indicating that, unlike PEPCase, induction of alternative oxidase protein cannot always be anticipated in response to low phosphorus. Consistent with this observation, and unlike bean or *G. sepium*, alternative pathway activity decreased in the tobacco leaves grown at low-phosphorus when compared with plants grown at high phosphorus (Fig. 1b).

It has long been suggested that when the cytochrome pathway activity is restricted or saturated, the alternative pathway will become more active (Vanlerbergh & McIntosh 1997; Millenaar et al. 1998). However, our results show that the response of the cytochrome pathway to low phosphorus does not necessarily correlate with the response of the alternative pathway (Fig. 1). In both bean and tobacco, the activity of the cytochrome pathway was inhibited by growth at low phosphorus as expected from an increased adenylate control on respiration. However, only in the case of bean did the activity of the alternative pathway increase.

**Figure 3.** Relationship between total respiratory (Total), cytochrome (Cyt) and alternative pathway (Alt) activities and cellular level of soluble carbohydrates in leaves of *P. vulgaris* plants grown at either high or low phosphorus. Correlation coefficients were statistically significant ($P < 0.05$) for total respiration and cytochrome pathway activity for plants grown at high phosphorus.
Conversely, alternative pathway activity increased in *G. sepium* grown at low phosphorus, but the cytochrome pathway was not inhibited (Fig. 1).

Respiratory activity is often correlated with carbohydrate levels (Penning de Vries, Witlage & Kremer 1979; Azcón-Bieto & Osmond 1983; Farrar 1985; Amthor 1994; Felitti & Gonzalez 1998). It has been suggested that at high carbohydrate levels there could be more alternative pathway activity if excess substrate saturates the cytochrome pathway (Azcón-Bieto et al. 1983; Lambers 1997). In plants grown at low phosphorus, the cytochrome pathway will be restricted by the rate of ADP phosphorylation and therefore is likely to be more readily carbohydrate saturated than plants grown at high phosphorus. Although starch levels in plants grown at low phosphorus declined in all three species, soluble sugar levels varied: decreasing in beans, increasing in tobacco and being unchanged in *G. sepium* (Table 2). To examine whether alternative pathway activity correlated with substrate concentration, respiratory activity was plotted against the soluble sugar concentration obtained from the same tissues (Figs 3 & 4). At high phosphorus, total respiratory and cytochrome pathway activities were significantly and positively correlated with sugar levels in bean (Fig. 3a) and tobacco (Fig. 4a). In contrast, alternative pathway activity was independent of cellular sugar levels (Figs 3a & 4a).

In plants grown at low phosphorus, the relationship between sugar levels and total respiration or cytochrome pathway activity was lost (Figs 3b & 4b), suggesting downstream control of the cytochrome pathway by ADP phosphorylation under these conditions. However, the activity of the alternative pathway, which can potentially avoid downstream control of respiration, was not still correlated with sugar concentration under low phosphate (Figs 3b & 4b). It is striking that the activity of the alternative pathway doubled when the level of soluble carbohydrate decreased in bean plants grown at low phosphorus (Fig. 1a & Table 2). In contrast, the activity of the alternative pathway in tobacco leaves grown at low phosphorus was essentially zero (Fig. 1b), despite the fact that soluble carbohydrate levels significantly increased (Table 2). This analysis does not support the concept that the operation of the alternative pathway is regulated primarily by excess carbohydrate (Lambers 1982; Azcón-Bieto et al. 1983), even under conditions where the cytochrome pathway is restricted.

Inhibition of the cytochrome pathway resulted in a lower rate of ATP formation in bean and tobacco leaves grown at low phosphorus (Table 3), suggesting that a reduction in respiration at low phosphorus may compromise ATP-requiring processes such as carbohydrate transport or tissue maintenance. Respiration of fully expanded leaves is often correlated with tissue N content, due to tissue maintenance costs (Amthor 1989; Cannell & Thornley 2000). Growth at low phosphorus resulted in increased N levels in bean but not in tobacco or *G. sepium* (Table 2). Therefore, respiratory ATP production expressed per unit N was substantially decreased in bean leaves grown at low phosphorus. However, in tobacco, the rates of ATP formation per unit N were the same in plants grown at low and high phosphorus (Table 3). These results indicate that lower respiration at low phosphorus in tobacco does not necessarily compromise the maintenance processes of the tissue. Tobacco leaves could have acclimated their metabolism upstream and downstream of mitochondrial electron transport leading to a decreased rate of respiration and avoiding over-reduction of the UQ pool. In bean, the cytochrome pathway alone apparently did not meet the ATP demand from maintenance processes at low phosphorus and as a consequence, the UQ pool could be susceptible to over-reduction if the alternative pathway is not active. Increased alternative pathway activity in bean grown at low phosphorus can alleviate over-reduction of the UQ pool and avoid formation of harmful reactive oxygen species (Purvis 1997; Wagner & Moore 1997; Maxwell, Wang & McIntosh 1999; Parsons et al. 1999).

In summary, our results indicate that whereas cytochrome pathway activity is regulated by carbohydrate, Pi and ADP availability, the regulation of alternative pathway activity is more complex. Alternative pathway activity is not under the direct control of carbohydrate level at either high or low phosphorus. Alternative pathway activity is also independent of the response of the cytochrome pathway to low phosphorus, so the alternative pathway does not always operate as a bypass for the cytochrome pathway under low phosphorus. Our results also suggest that alternative pathway activity may increase...
when reduced respiration at low phosphorus is not sufficient to meet the energetic demands of tissues. Further studies are needed to better understand the combination of factors that ultimately contribute to the activity of the alternative pathway across different plant species.

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